The role of megsin, a serine protease inhibitor, in diabetic mesangial matrix accumulation

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In diabetic nephropathy decreased activities of matrix metalloproteinase (MMP)-2, MMP-9 and plasmin contribute to mesangial matrix accumulation. Megsin, a novel member of the serine protease inhibitor superfamily, is predominantly expressed in mesangial cells and is up-regulated in diabetic nephropathy and its overexpression spontaneously induces progressive mesangial expansion in mice. High-glucose stimulated megsin mRNA expression in an *in vivo* model of type II diabetic nephropathy as well as *in vitro* in cultured mesangial cells. Megsin potentially inhibits total enzymatic activities of MMP-2 and -9 and plasmin, indicating decreased degradation of mesangial matrix. A specific monoclonal anti-megsin neutralizing antibody restored MMP activity in a transforming growth factor-β independent manner. Our study suggests that the mesangial matrix accumulation caused by hyperglycemia in diabetes might be due at least in part to up-regulation of megsin which can inhibit plasmin and MMP activities.

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Diabetic nephropathy is characterized by a progressive mesangial expansion mainly due to the accumulation in the extracellular matrix (ECM) of collagen IV, laminin, fibronectin, proteoglycans, and other matrix proteins.[1–6] This accumulation is due to a disturbed balance between synthetic and degradative pathways.[2,3]

Two major proteolytic pathways degrade glomerular ECM, namely, matrix metalloproteinases (MMPs) and plasmin. MMP-2 and MMP-9 cleave collagen IV, laminin, and fibronectin, and the serine protease plasmin degrades laminin and fibronectin.[7–9] MMP-2 and MMP-9 are synthesized in latent forms, unable to effect proteolysis.[10,11] Plasmin also converts inactive into active MMPs.[10,12,13] It is now recognized that in diabetic nephropathy, glomerular ECM accumulation is mediated by a plasmin/MMPs cascade, that is, decreased activities of plasmin and MMP-2 and MMP-9.[7–10,14]

To elucidate the pathogenesis of diabetic kidney disease, a functional quantitative analysis of the genome in cultured human mesangial cells was performed and the expression levels of a variety of mesangial transcripts with those observed in other non-renal cells were compared.[15]

Megsin, a novel member of the serine protease inhibitor (serpin) superfamily, is predominantly expressed in mesangial cells.[16] By *in situ* hybridization and immunohistochemistry, megsin gene and proteins were mesangial cells.[16–18] Its overexpression in transgenic mice induces progressive mesangial matrix accumulation.[19] Although its expression is ubiquitous, its pathogenic effects are restricted to glomeruli. Cross-breeding of this mouse model with RAGE (the receptor for advanced glycation end products)/inducible nitric oxide synthase double transgenic diabetic mice has a triple transgenic model characterized by a severe mesangial matrix accumulation, including nodular lesions, similar to those observed in humans.[20]

In the present study, we used a neutralizing antibody against megsin activity to investigate the role of megsin in the glomerular ECM accumulation characteristic of diabetic nephropathy. *In vitro* and *in vivo* data demonstrate that hyperglycemia upregulates megsin, which, in turn, inhibits

plasmin and MMPs in a transforming growth factor (TGF)-β-independent manner. This chain of events potentially contributes to ECM accumulation in diabetic glomeruli.

RESULTS

Megsin expression in diabetic SHR/NDmcr-cp rats

Spontaneously hypertensive/NIH-corpulent (SHR/NDmcr-cp) rats are a well-known type II diabetic rat model.[21] At the age of 17 weeks, SHR/NDmcr-cp rats exhibited a wide range of metabolic abnormalities, that is, hyperglycemia, dyslipidemia, and hypertension (Table 1) in comparison with control Wistar-Kyoto (WKY) rats. A real-time PCR analysis in the renal cortex revealed a significant increase in the megsin mRNA expression in diabetic SHR/NDmcr-cp rats, compared with WKY rats ($P<0.01$, Figure 1a).

Table 1 | Biochemical and physiological data of the experimental rats aged 17 weeks

	WKY	DM
HbA1c (%)	3.0 ± 0.1	6.8 ± 0.3***
Total cholesterol (mg per 100 ml)	141 ± 3	163 ± 4**
Triglyceride (mg per 100 ml)	81 ± 13	720 ± 61***
Systolic blood pressure (mm Hg)	129 ± 2	165 ± 4***

WKY, Wistar-Kyoto.
**$P<0.01$, ***$P<0.001$ vs WKY. Data are represented as the mean ± s.e.m.

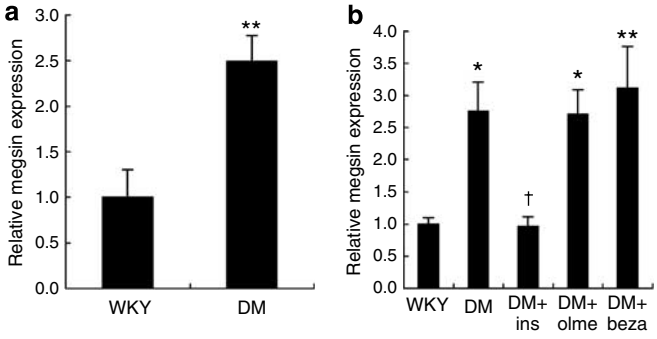

Figure 1 | Upregulation of megsin mRNA expression in diabetic rat kidney. (**a**) Megsin mRNA expression in kidney tissues at the age of 17 weeks in diabetic SHR/NDmcr-cp rats (DM) and control WKY rats (WKY). (**b**) Megsin mRNA expression at age the of 39 weeks in diabetic SHR/NDmcr-cp rats (DM), DM rats on insulin (DM + ins), rats on olmesartan (DM + olme), rats on bezafibrate (DM + beza), and control WKY rats (WKY). The mRNA expression of megsin was highly upregulated in diabetic kidneys, and was significantly decreased only by insulin treatment. *$P<0.05$, **$P<0.01$ vs WKY; †$P<0.05$ vs DM.

To ascertain the metabolic factors involved in the upregulation of megsin expression, we assessed megsin expression in diabetic SHR/NDmcr-cp rats treated with several drugs, that is, insulin, olmesartan, and bezafibrate. The biological data of experimental animals at the end of the study (aged 39 weeks) are summarized in Table 2. All drugs significantly reduced total cholesterol and triglycerides levels at the end of study. Insulin, but not other agents, significantly improved hyperglycemia, whereas only olmesartan achieved a better blood pressure control. The increase in megsin expression observed in SHR/NDmcr-cp rats was significantly lowered only by insulin treatment ($P<0.05$, Figure 1b). These findings suggest that hyperglycemia, but not hypertension and hyperlipidemia, is associated with the upregulation of megsin expression.

Megsin expression *in vitro* in RMCs

We next examined whether high glucose induces megsin expression *in vitro* in cultured rat mesangial cells (RMCs). As expected from the *in vivo* results, megsin mRNA expression was significantly increased in RMCs cultured in high glucose, compared with low glucose ($P<0.05$, Figure 2). High osmolarity did not affect megsin expression.

Effects of megsin on plasmin and MMP activities *in vitro*

Megsin inhibits an enzymatic activity of plasmin, a well-known enzyme that converts inactive into active MMPs.[10,19] We thus tested the interrelation between megsin and MMP activities *in vitro* in RMCs. Incubation for 72 h in a megsin-fortified medium significantly decreased not only plasmin activity (Figure 3a, $P<0.001$) but also total MMP-2 and MMP-9 activities (Figure 3b, $P<0.05$).

We also tested a direct interrelation between megsin and MMP activities. In cell-free assay system, megsin did not affect MMPs activity (1.00 ± 0.02 of MMP vs 0.98 ± 0.01 of MMP and megsin). These data thus suggest that megsin indirectly links to MMP activation via the inhibition of plasmin enzymatic activity.

Characterization of anti-megsin monoclonal antibody MS18a

To understand the role played by endogenous megsin, an anti-megsin monoclonal antibody named MS18a was developed. Epitope mapping analysis showed that the anti-megsin antibody MS18a was directed against the residues NIVEKQ located in the reactive loop site of human megsin protein[16] (Table 3). The residues NIVEKL in rat megsin protein correspond to the residues NIVEKQ in the human megsin

Table 2 | Biochemical and physiological data of the experimental rats aged 39 weeks

	WKY	DM	DM+ins	DM+olme	DM+beza
HbA1c (%)	3.3 ± 0.0	5.3 ± 0.3***	3.3 ± 0.2†††	5.3 ± 0.2***	5.2 ± 0.3***
Total cholesterol (mg per 100 ml)	128 ± 3	186 ± 10***	132 ± 8†††	141 ± 5†††	132 ± 7†††
Triglyceride (mg per 100 ml)	39 ± 2	407 ± 44***	221 ± 42**,††	259 ± 30***,†	239 ± 23***,††
Systolic blood pressure (mm Hg)	139 ± 3	170 ± 4**	178 ± 6***	115 ± 6*,†††	173 ± 4***

WKY, Wistar-Kyoto.
*$P<0.05$, **$P<0.01$, ***$P<0.001$ vs WKY; †$P<0.05$, ††$P<0.01$, †††$P<0.001$ vs DM. Data are represented as the mean ± s.e.m.

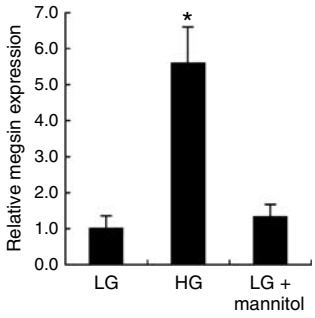

Figure 2 | Megsin expression *in vitro* in rat mesangial cells (RMCs). Cells were exposed to 5 mM glucose (LG), 25 mM glucose (HG), or 5 mM glucose + 20 mM mannitol (LG + mannitol) for 72 h. Megsin mRNA expression was significantly higher in RMCs cultured in a high-glucose medium. *$P<0.05$ vs LG.

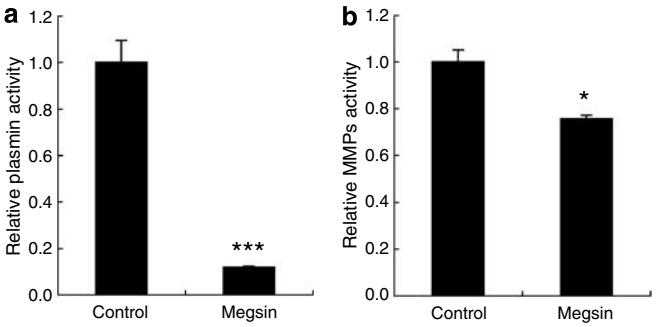

Figure 3 | Effects of megsin on plasmin and MMPs activities. (**a**) The activity of plasmin, and (**b**) total MMP-2 and MMP-9 activities in RMCs cultured in a megsin-fortified medium (low-glucose). Megsin significantly decreased the plasmin activity, and the total MMP-2 and MMP-9 activities. *$P<0.05$, ***$P<0.001$ vs control.

reactive loop site.[22] We confirmed that MS18a was also directed against rat megsin peptide containing NIVEKL residues (bottom of Table 3). These data suggest that the epitope of MS18a is the residues NIVEKQ/NIVEKL.

We further validated the neutralizing activity of anti-megsin monoclonal antibody MS18a using cell-free assay system. The reduction of plasmin activity by human megsin protein was increased by anti-megsin antibody MS18a ($P<0.001$), in contrast to control IgG (Figure 4). The blocking peptide corresponding to the reactive loop site of rat megsin competitively reduced plasmin activity restored by MS18a, which was found to be significant ($P<0.001$). These findings revealed that MS18a has the potential to neutralize not only human but also rat megsin enzymatic activity against plasmin.

Role of megsin in RMCs under high-glucose condition

To elucidate the role of high-glucose-induced megsin, we investigated MMPs activities *in vitro* in RMCs using anti-megsin monoclonal neutralizing antibody MS18a. As shown in Figure 3, megsin mRNA expression was upregulated under high-glucose conditions. The total MMP-2 and MMP-9 activities were significantly decreased in RMCs cultured in a high-glucose medium, compared with low-glucose ($P<0.05$, Figure 5). MS18a significantly ($P<0.01$) increased total MMP-2 and MMP-9 activities in RMCs cultured in a high-glucose medium, in contrast to control IgG. We also confirmed, in cell-free direct assay system, that MS18a did not affect MMP activity directly (1.00 ± 0.01 of MMP vs 1.02 ± 0.02 of MMP and MS18a). These findings suggest that hyperglycemic conditions reduce MMP activities, at least in part, through the megsin modulation.

Table 3 | Epitope mapping of anti-megsin monoclonal antibody MS18a

Peptide sequence	MS18a antibody activity
TAATGSNIVEKQLPQST	–
AATGSNIVEKQLPQST	–
ATGSNIVEKQLPQST	–
TGSNIVEKQLPQST	–
GSNIVEKQLPQST	–
SNIVEKQLPQST	–
NIVEKQLPQST	–
IVEKQLPQST	+
VEKQLPQST	++
TAATGSNIVE	++
TAATGSNIVEK	++
TAATGSNIVEKQ	–
TAATGSNIVEKQL	–
TAATGSNIVEKQLP	–
TAATGSNIVEKQLPQ	–
TAATGSNIVEKQLPQS	–
NIVEKQ	–
TAATESNIVEKLLPES	–

++, antibody activity similar to non-peptide control; +, about half of antibody activity; –, complete loss of antibody activity.

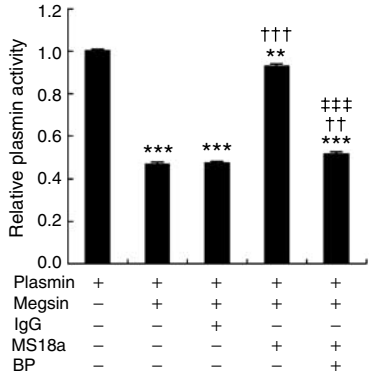

Figure 4 | Characterization of anti-megsin monoclonal antibody MS18a. Cell-free assay for validation of neutralizing activity of MS18a. BP, blocking peptide, corresponding to the rat megsin reactive loop site. MS18a significantly neutralized megsin enzymatic activity against plasmin. **$P<0.01$, ***$P<0.001$ vs plasmin only (far left); $^{\dagger\dagger}P<0.01$, $^{\dagger\dagger\dagger}P<0.001$ vs plasmin and megsin (second left); $^{\ddagger\ddagger\ddagger}P<0.001$ vs MS18a (second right).

Relationship between megsin and TGF-β

TGF-β is a pivotal factor in the development of glomerular ECM accumulation in diabetic nephropathy.[3,10,23] The interrelation between TGF-β and megsin was therefore evaluated. TGF-β did not affect the mRNA expression of

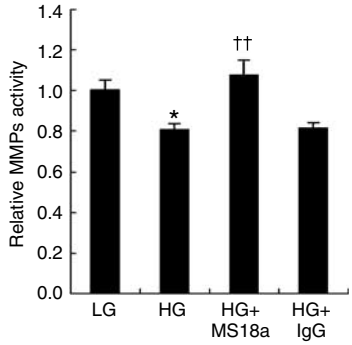

Figure 5 | Role of megsin in mesangial cells under high-glucose condition. RMCs were exposed to 5 mM glucose (LG), 25 mM glucose (HG), HG with anti-megsin neutralizing antibody MS18a, or HG with control IgG for 72 h. Lowered total MMP-2 and MMP-9 activities in HG were significantly increased via neutralizing of megsin activity by MS18a. $^{*}P<0.05$ vs LG, $^{\dagger\dagger}P<0.01$ vs HG.

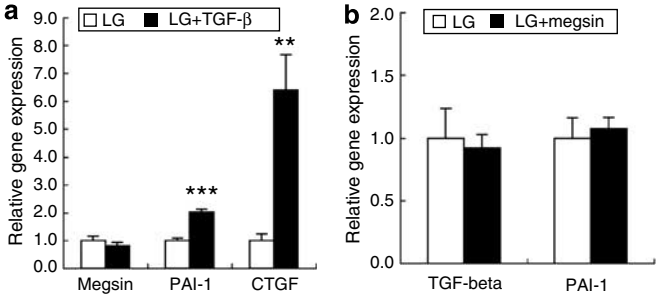

Figure 6 | Correlation between megsin and TGF-β. (**a**) Effects of TGF-beta on genes expressions of megsin, PAI-1, and connective tissue growth factor (CTGF), and (**b**) the effect of megsin on TGF-β and PAI-1 in RMCs cultured in low-glucose (LG) medium. There is no correlation between megsin and TGF-β. $^{**}P<0.01$, $^{***}P<0.001$ vs LG control.

megsin in RMCs, in contrast to plasminogen activator inhibitor (PAI)-1 and connective tissue growth factor, both well known TGF-β-induced genes[24,25] (Figure 6a). PAI-1 itself plays an important role in glomerular ECM accumulation as well as TGF-β.[26]

Megsin did not affect the mRNA expressions of TGF-β and PAI-1 (Figure 6b), and did not modify the enzymatic activity of PAI-1 (1.00 ± 0.02 of PAI-1 vs 1.04 ± 0.01 of PAI-1 and megsin). These findings suggest that the expression of megsin is independent of the production of TGF-β and PAI-1.

DISCUSSION

Megsin is implicated in the development of renal diseases, including diabetic nephropathy, but its biological function remains elusive. Using binding and functional assays *in vitro*, we previously reported that megsin is a proteinase inhibitor whose biological substrate is plasmin.[19] We also observed the upregulation of megsin in human diabetic nephropathy.[16,27] In the present study, we now demonstrate that high glucose *per se* induces megsin expression both *in vivo* in a model of type II diabetic nephropathy and *in vitro* in cultured mesangial cells in which it also inhibits total MMP-2 and MMP-9 activities and plasmin enzymatic activity. These data suggest that the reduction of protease activities decreases the degradation of glomerular ECM and results in mesangial expansion in diabetic nephropathy. This is consistent with the aggravation of diabetic nephropathy with Kimmelstiel–Wilson-like nodules in the triple transgenic mice over-expressing inducible nitric oxide synthase, RAGE, and megsin.[20] The current study clarifies, at the molecular level, the pathophysiogical role of megsin upregulation in diabetic nephropathy.

Previously, we demonstrated that the activator protein (AP)-1 binding site is within the 5′-flanking region of megsin gene and that it potentially regulates megsin expression.[28] The transcription factor AP-1 regulates various gene expressions through its binding site. AP-1 binding increases in mesangial cells under hyperglycemic conditions.[29] Hence, megsin is upregulated under hyperglycemic conditions through increased AP-1 binding. The upregulation of megsin is due to a metabolic effect of high glucose, as osmolarity control with mannitol failed to induce megsin expression in our experiments. However, glucose level is not the sole regulator of megsin expression. Megsin is upregulated not only in diabetic conditions but also in non-diabetic glomerulonephritis, that is, IgA nephropathy.[16,17] The mechanism underlying the upregulation of megsin in IgA nephropathy remains elusive, and requires further studies to elucidate its molecular mechanisms.

Under normal circumstances, MMP activities are tightly regulated.[10] Our *in vitro* studies with the anti-megsin neutralizing antibody demonstrated that megsin decreased the active forms of MMP-2 and MMP-9 under hyperglycemic conditions via the inhibition of plasmin enzymatic activity. This decrease plays a role in glomerular ECM overaccumulation, which resulted in diabetic glomerulosclerosis.[7–10,14,30,31] Indeed, angiotensin-converting enzyme inhibitors, statins, and estradiol, restore the reduced activities of MMPs and provide renoprotection.[32–35] Under hyperglycemic conditions, however, the suppression of MMPs activity was partial in contrast to the drastic upregulation of megsin, suggesting that factors other than megsin also contribute to this effect.

All serpins including megsin share a common structure, that is,[36] a β-sheet-rich body and an exposed mobile reactive loop, which functions as a pseudosubstrate for the target protease (for example, plasmin for megsin). The inhibitory mechanism of serpin rests on the unique conformational flexibility, and the insertion of the cleaved reactive center loop of the serpin into the large central β-sheet, leading to the formation of a stable proteinase–serpin complex. Our *in vitro* studies demonstrate that MS18a specifically recognized the residues NIVEKQ/NIVEKL, comprised in the reactive loop site of human, chimpanzee (XP_524011.2), dog (XP_541072.2), rat, and mouse megsin.[16,22] It thus restores the access of plasmin to cleave the reactive center loop of megsin. Neutralizing antibodies against PAI-1, another

member of the serpin superfamily, also recognize the corresponding portion of the reactive loop site.[37]

TGF-β plays a pivotal role in the ECM accumulation in diabetic glomeruli.[3,10,23] It controls MMP activities by modulating transcription; by post-translational modification, that is, the inhibition of plasmin generation via PAI-1; and by the upregulation of tissue inhibitors of metalloproteinases-1.[3,10,23,24,38] The mechanism of the inhibition of MMP activities by megsin is independent of TGF-β, in contrast to the protective mechanism of PAI-1 deficiency in a model of diabetic nephropathy, which appears to be mediated by TGF-β.[39] A hypothetical schema depicted in Figure 7 tentatively integrate these various observations to understand the genesis of diabetic glomerular lesions. The inhibition of MMP activities by megsin and TGF-β are independent from each other, so that megsin could become a novel therapeutic target to prevent diabetic glomerular ECM accumulation. The future availability of megsin inhibitors should also open new therapeutic avenues.

In conclusion, high glucose induces the upregulation of megsin both *in vitro* and *in vivo*. Functional studies show that megsin inhibits the activities of plasmin and MMPs independent of TGF-β. The upregulation of megsin thus links to pathological changes in diabetic nephropathy. The current study clarifies, for the first time, at the molecular level, the role of megsin upregulation in diabetic nephropathy.

MATERIALS AND METHODS

Type II diabetic rats

All animal experiments were performed in accordance with the guidelines of the Committee on Ethical Animal Care and Use of Tokai University.

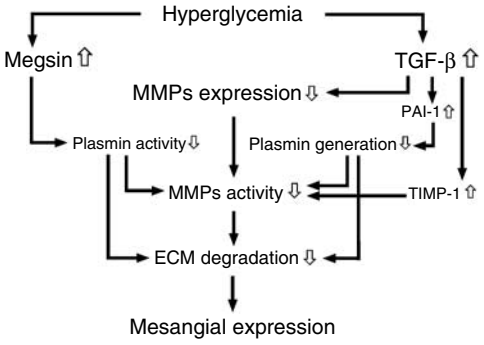

Figure 7 | Hypothetical schema for diabetic glomerular ECM accumulation. Megsin is a potent suppressor of glomerular MMP activities in diabetes, independently of TGF-β.

Ten male SHR/NDmcr-cp rats and five WKY rats were purchased from SLC (Shizuoka, Japan). They were housed in individual cages under a temperature- and light-controlled environment in an accredited animal care. At the end of the study, at the age of 17 weeks, they were killed and blood samples were obtained.

Separately from the above experiment, a total of 39 male SHR/NDmcr-cp rats and 10 WKY rats were purchased from SLC. SHR/NDmcr-cp rats, aged 13 weeks, were randomly divided into four groups, namely, Group 1 received vehicle orally (DM, $n=10$), Group 2 received insulin subcutaneously (30 units/kg, twice a day) (DM + ins, $n=9$), Group 3 received olmesartan orally (5 mg/kg) (DM + olme, $n=10$), and Group 4 received bezafibrate orally (25 mg/kg) (DM + beza, $n=10$). WKY rats served as a control group (WKY, $n=10$). The drug treatment lasted for 26 weeks. At the end of the study, at the age of 39 weeks, they were killed and blood samples were obtained.

The following methods were used: HbA1c by the DCA2000 (Bayer Diagnostics, Pittsburgh, PA, USA), plasma total cholesterol and triglycerides by the automatic analyzer (Hitachi Automatic Clinical Analyzer 7170, Hitachi Science Systems, Ibaraki, Japan), and systolic blood pressure in conscious rats by the tail-cuff method. Total RNA was extracted from renal cortex tissues for gene expression analysis with the RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions.

Gene expression analysis

Real-time PCR was performed by a previously described method[40] with the One Step RT-PCR Kit (Takara Bio Inc., Shiga, Japan), SYBR Green I reagent (Cambrex Bio Science, Rockland, ME, USA), and iCycler PCR system (Bio-Rad Laboratories, Hercules, CA, USA) for the evaluation of the mRNA expression of megsin, TGF-β, PAI-1, connective tissue growth factor, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primer sequences are listed in Table 4. GAPDH mRNA expression was used for the normalization of these genes expressions. Data are presented as relative values.

Culture of RMCs

RMCs, a cultured cell line originating from mesangial cells of male Sprague–Dawley rats, were purchased from ATCC (Manassas, VA, USA). For the present experiments, cells obtained after 10 and 12 passages were used. RMCs were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum and 0.4 mg/ml G-418.

Megsin expression in RMCs

RMCs were maintained in DMEM containing either 5 or 25 mM D-glucose with 10% fetal bovine serum for 72 h ($n=6$). The effect of hyperosmolality was assessed in RMCs cultured in DMEM containing 5 mM D-glucose supplemented with 20 mM mannitol. For gene expression analysis, total RNA was isolated from RMCs with the RNeasy Mini Kit according to the manufacturer's instructions.

Table 4 | Primers for real-time PCR

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
Megsin	AGAATTTGGCTTCGACTTATTCAGAGAGATGG	ATGACAGCTGATGAGCTGAGGCTGCTGTCCCCC
TGF-β	TGCGCCTGCAGAGATTCAAG	AGGTAACGCCAGGAATTGTTGCTA
PAI-1	GCCCAGCATTCAGCCTTTG	AAGACTTTGCTGAGTGAAGGCGTAG
CTGF	CACCCGGGTTACCAATGACAA	AGCCCGGTAGGTCTTCACACTG
GAPDH	GACAACTTTGGCATCGTGGA	ATGCAGGGATGATGTTCTGG

CTGF, connective tissue growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TGF-β, transforming growth factor-β.

Recombinant human megsin protein

The entire coding sequence of human megsin cDNA was ligated into the His-tag fusion protein vector pET28a (Novagen Inc., Madison, WI, USA). The His-tagged megsin protein was expressed in *Escherichia coli*, and affinity purified by Ni Sepharose 6 Fast Flow (GE Healthcare UK Ltd., Buckinghamshire, UK) according to the manufacturer's instructions.

Effects of megsin on plasmin and MMP activities in RMCs

RMCs were cultured in 96-well plates and grown to subconfluence. They were subsequently washed and incubated with serum-starved DMEM (5 mM D-glucose) containing 0.05 U/ml plasminogen (Sigma-Aldrich Inc., St Louis, MO, USA) with or without recombinant human megsin (100 μg/ml) for 72 h ($n=6$). After incubation, culture supernatant was collected for the measurement of the activities of plasmin and gelatinases (total activities of MMP-2 and MMP-9). This experimental system, that is, activation of MMPs in cultured mesangial cells by exogenous plasminogen addition, has been previously reported.[23,38]

Measurement of MMP and plasmin activities

Plasmin activity in medium obtained from RMCs was determined as below. 50 μl of each medium sample were incubated with 0.3 mM of a plasmin colorimetric substrate, S-2403 (Daiichi Pure Chemicals, Co., Ltd., Tokyo, Japan) for 60 min, and absorbance was measured at 405 nm. The total MMP-2 and MMP-9 activities (gelatinase activity) in medium from RMCs were determined by a commercially available kit LL-20002 (Life Laboratory Company, Yamagata, Japan), according to the manufacturer's instructions. Results were corrected for cell number, respectively. Cell number was assessed using tetrazolium salt WST-1 (Dojindo Laboratories, Kumamoto, Japan), according to the manufacturer's instructions. Data are presented as the relative values.

Direct effects of megsin on MMP activities

Cell-free direct assay was performed as below: 5 mU of included standard MMP-9 protein was incubated with or without 10 μg of recombinant megsin protein, and its activity was measured by the LL-20002 kit (Life Laboratory Company) as above ($n=4$). Data are presented as relative values.

Development of anti-megsin monoclonal antibody

Male BALB/c mice (Charles River Japan, Kanagawa, Japan) were immunized by recombinant human megsin protein. Hybridomas expressing neutralizing antibodies were generated by a combined polyethylene glycol electrofusion of spleen cells from immunized mice and SP2 myeloma cells. They were screened by enzyme-linked immunosorbent assay on plates coated with human megsin protein. After incubation with each antibody, the wells were incubated with horseradish peroxidase-conjugated anti-mouse IgG antibody (Chemicon Inc., Temecula, CA, USA). For the development, the wells were incubated with a reaction solution containing *o*-phenylenediamine dihydrochloride, and absorbance was measured at 490 nm. Positive hybridomas were subcloned by limiting dilution. A clone named MS18a was eventually identified among these clones for subsequent screening. Antibody isotyping showed that MS18a is IgG_1 molecule.

Epitope mapping

The anti-megsin monoclonal antibody MS18a was pre-incubated with an excess of the several synthetic human megsin peptides (Fuso Pharmaceutical Industries, Osaka, Japan) (Table 3) for 2 h. They were subsequently evaluated by enzyme-linked immunosorbent assay on plates coated with human megsin protein as above.

Validation of neutralizing activity of MS18a

For validation of neutralizing activity of MS18a, cell-free assay was performed as below. A total of 4 μg of human megsin protein was pre-incubated with 10 μg of control mouse IgG, 10 μg of MS18a, or 10 μg of MS18a pre-incubated with an excess of the blocking peptide corresponding to the reactive loop site of rat megsin, that is, TAATESNIVEKLLPES (Sigma-Aldrich). They were subsequently incubated with 250 ng of plasmin (Sigma-Aldrich) for 30 min ($n=3$). The wells were incubated with S-2403 substrate, and absorbance was measured at 405 nm. Data are presented as relative values.

Neutralization of megsin in RMCs

RMCs were cultured in 96-well plates and grown to subconfluence. They were subsequently washed and incubated with DMEM (5 or 25 mM D-glucose with 10% fetal bovine serum) containing either 100 μg/ml MS18a or control mouse IgG for 72 h ($n=8$). After incubation, the culture supernatant was collected for the measurement of the total MMP-2 and MMP-9 activities by the LL-20002 kit (Life Laboratory Company) as above. Data are presented as relative values.

Direct interrelation between MS18a and MMP activities

Cell-free direct assay was performed as below: 5 mU of included standard MMP-9 protein was incubated with or without 10 μg of anti-megsin monoclonal antibody MS18a, and its activity was measured by the LL-20002 kit (Life Laboratory Company) as above ($n=4$). Data are presented as relative values.

Effects of megsin and TGF-β on genes expressions

RMCs were cultured in 96-well plates and grown to confluence. They were subsequently washed and incubated with serum-starved DMEM (5 mM D-glucose) containing TGF-β (5 ng/ml, Sigma-Aldrich) or megsin (100 ng/ml) for 24 h ($n=6$). Total RNA was isolated from RMCs with the RNeasy Mini Kit, and mRNA expression was evaluated by real-time PCR.

Effects of megsin on PAI-1 activity

Cell-free direct assay was performed as below. The PAI-1 activity was determined by a commercially available kit (HYPHEN BioMed, Neuville-sur-Oise, France). A total of 1 ng of included standard PAI-1 protein was incubated with or without 25 ng of recombinant megsin protein, and its activity was measured according to the manufacturer's instructions ($n=4$). Data are presented as the relative values.

Statistical analysis

All data are reported as the mean ± s.e.m. Statistical analysis was performed with SPSS for Windows version 15.0 (SPSS, Chicago, IL, USA). Comparisons between two groups were performed using an unpaired *t*-test. For multiple comparisons, one-way analysis of variance and Tukey's *post hoc* test were performed. $P<0.05$ was considered significant.

DISCLOSURE

All the authors declared no competing interests.

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